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Gene therapy using non-viral peptide vector in a canine systemic lupus erythematosus model☆

Eun-Wha Choi[a,c], Il-Seob Shin[b,c], Hwa-young Youn[b,c], Dae-Yong Kim[c,d], Hang Lee[c,e], Young-Jin Chae[f,**], Chang-Woo Lee[a,*]

[a]*Department of Veterinary Clinical Pathology, College of Veterinary Medicine, Seoul National University, San 56-1,Shillim-Dong, Kwanak-Gu, Seoul 151-742, South Korea*
[b]*Department of Veterinary Internal Medicine, College of Veterinary Medicine, Seoul National University, Seoul 151-742, South Korea*
[c]*Department of Agricultural Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, South Korea*
[d]*Department of Veterinary Pathology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, South Korea*
[e]*Department of Veterinary Biochemistry, College of Veterinary Medicine, Seoul National University, Seoul 151-742, South Korea*
[f]*Ra-science, Ilsan-Gu, Goyang-City, Kyunggi-Do 411-380, South Korea*

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Abstract

Although viral vectors are commonly used for therapeutic gene delivery, their applications are limited due to their specific cell membrane receptor-mediated infection and host immune response. In the present study, we constructed a non-viral peptide vector and applied it in the treatment of experimentally induced systemic lupus erythematosus-like disease in dogs. For therapeutic gene construction, the extracellular domain of canine CTLA-4, and the CH2–CH3 domains of canine immunoglobulin alpha constant region were inserted between the cytomegalovirus promoter and poly-adenylation sequence of bovine growth hormone. The constructed therapeutic gene was ligated to the non-viral synthetic peptide vector and was applied to systemic lupus erythematosus-like disease induced dogs. After gene therapy, clinical signs of systemic lupus erythematosus were reduced dramatically: the anti-nuclear antibody titers and urine protein/creatinine ratios were recovered to normal values, and the skin regained its normal histological features. The peptide vector did not show either tissue specific tropism or host induced immune response.

Keywords: Non-viral peptide vector; CTLA-4; Systemic lupus erythematosus; Costimulation; Dog

☆ Address to where requests for peptide vector should be sent: pellucida@korea.com.
* Corresponding author. Tel.: +82 2 880 1273; fax: +82 2 880 1212.
** Co-corresponding author.
E-mail address: anilover@snu.ac.kr (C.-W. Lee).

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1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune syndrome defined by clinical and serological features, including arthritis, glomerulonephritis, dermatitis and autoantibodies. In SLE, long-term disease control and the minimization of iatrogenic risk usually require adjunctive therapies that target the more fundamental immunoregulatory disturbances of lymphoid cells (Balow et al., 2000). Antigenic stimulation of T cells generally requires the presence of two signals provided by an antigen presenting cell (APC) (Clark and Ledbetter, 1994; Gimmi et al., 1993; Mueller et al., 1989). The first signal is mediated via the T cell receptor/CD3 complex and an antigenic peptide presented by a major histocompatibility complex (MHC) molecule, which is required for antigenic stimulation of T cells. The second signal is mediated via B7:CD28 costimulation which induces proliferation of T cells. B7 is the counter-receptor, which has two ligands, CD28 and CTLA-4, expressed on T lymphocytes (Linsley et al., 1990; Linsley et al., 1991). The first ligand, termed CD28, is constitutively expressed on T cells, and after ligation, induces IL-2 secretion and proliferation (June et al., 1990). The second ligand, termed CTLA-4, is homologous to CD28 and appears on T cells after activation (Freeman et al., 1992). Since the affinity of B7 for CTLA-4 is 20 times higher than that for CD28, CTLA4Ig fusion protein is the most effective reagent to inhibit the B7:CD28 costimulatory pathway (Lenschow et al., 1992; Turka et al., 1992). Blockade of the B7:CD28 costimulatory interactions with soluble CTLA4Ig fusion protein has been shown to inhibit humoral immunity (Linsley et al., 1992), graft rejection (Lenschow et al., 1992; Lin et al., 1993), and graft versus host disease (Blazar et al., 1994), and to ameliorate autoimmune diseases (Finck et al., 1994) including experimental autoimmune encephalomyelitis, diabetes, and collagen-induced arthritis. In the gene therapy, the ideal vector should have the following features: it should be delivered to various tissues and have a long duration of expression, but it should not evoke immune response, not have size restriction, not have toxic effects, and not be integrated into the host's genome. However, conventional viral vectors have not been able to satisfy the above demands (Bordignon et al., 1995; Cartmell et al., 1999; Dai et al., 1995; Laquerre et al., 1999; Lowenstein et al., 1994; Stone et al., 2000; Xiao et al., 1996; Worgall et al., 1997).

In this experiment, we designed the peptide vector to overcome conventional problems of viral vectors and applied the peptide vector encoding canine CTLA4Ig fusion therapeutic gene to an experimental canine SLE model.

2. Materials and methods

2.1. Induction of an SLE-like disease in dogs

Eight male dogs (mixed breed, age: 4–6 years) were determined to be healthy and have normal renal function as assessed by physical examination, complete blood count (CBC), serum biochemistry analysis and urinalysis. The dogs were housed separately in each cage and maintained on commercial dry dog food and water provided ad libitum. The animals were acclimatized at least 1 week prior to use in any experiment. We utilized heparan sulfate (HS) to induce an SLE-like disease in eight male dogs. HS is the major glycosaminoglycan of glomerular basement membrane. Autoimmunity to HS has been suggested to be responsible for the induction of tissue damage and kidney dysfunction in SLE in both in vitro (Faaber et al., 1986) and in vivo (Naparstek et al., 1990; Ofosu-Appiah et al., 1998). Two hundred micrograms of HS in 2 ml PBS was emulsified in 2 ml of complete Freund's adjuvant (CFA; Sigma) containing *Mycobacterium tuberculosis* strain H37Ra. The inoculum (4 ml) was applied to four injection sites at the back and flanks of the dogs. Booster injections were given at 2, 4, and 6 weeks using HS emulsified in incomplete Freud's adjuvant (IFA). During the experiment, serum biochemical parameters and UP/Cs were measured weekly. All eight dogs developed SLE-like disease. Before the therapeutic gene construction, four of eight SLE-induced dogs died. Two of four surviving dogs were treated by peptide vector encoding the therapeutic gene and the other two dogs were used as control.

2.2. Construction of the therapeutic gene

We constructed a therapeutic gene (Fig. 1) composed of the extracellular domain of canine

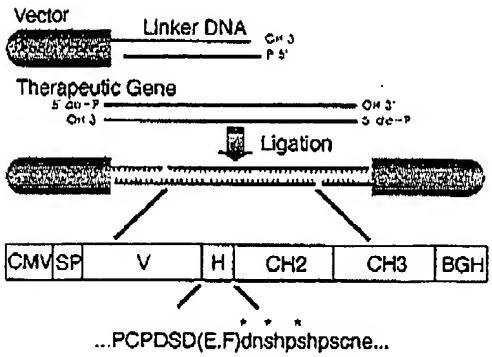

Fig. 1. Construction of the therapeutic gene. Map of the therapeutic gene construct. The cDNA construct encoding the indicated portions of oncostatin M (SP), CTLA-4 (V), and canine IgA (H, CH2, and CH3) was constructed as described in Section 2. Sequences displayed show the hinge (H) of IgA. The amino acid in parentheses was introduced during construction. Asterisks denote cysteine to serine mutations introduced in the IgA hinge region. CMV promoter and BGH poly-A signal obtained from pcDNA3.1(+) (Invitrogen, USA).

CTLA-4 to inhibit the B7:CD28 costimulatory pathway and the CH2–CH3 domains of the canine immunoglobulin alpha constant (IGHAC) region to prolong the half-life of therapeutic protein in vivo. The cytomegalovirus (CMV) promoter for the high level expression, the human oncostatin M signal sequence for the secretion to body fluid, and the polyadenylation sequence of bovine growth hormone for efficient termination of transcription were ligated to the therapeutic gene. The fusion sequence of oncostatin M, CTLA-4 extracellular domain and the CH2–CH3 domains of the IGHAC region was ligated to *Hin*dIII and *Xba*I sites in pcDNA3.1(+) (Invitrogen, USA). Primer pairs were prepared: CMV-F 5′-GCCAGATATACGCGTTGACAT-3′ and BGH-R 5′-GCTTAATGCGCCGCTACA-3′. With these primers, approximately 2213 bp fragments were amplified using pcDNA 3.1(+)/CTLA4Ig as templates.

2.3. Peptide vector construction

The delivery vector includes leader peptide and linker DNA (Fig. 1). Leader peptide has 15 amino acids, which are designed to have functions for membrane fusion and penetration. The linker DNA bridges the leader peptide and a therapeutic gene.

The sequences of leader peptide, linker-C, and linker-2 were as follows:

Leader peptide: Ac-Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gly-Arg-Arg-Cys

Linker-C: 5′-Cys–OO–CTAATACGACTCACTAT-3′ (–OO–: ester bond)

Linker-2: 3′-GATTATGCTGAGTGAT-5′

The N-terminal amino group was replaced with an acetyl group to remove molecular activity. Leader peptide and linker-C were conjugated with a disulfide bond by incubating in an S–S bond buffer (50 mM Tris, 0.1 mM EDTA, 10 mM DTT, pH 10.5) at 37 °C for 1 h. After this procedure, linker-2 was added and incubated at 60 °C for 30 min for the hybridization between linker-C and linker-2. The peptide vector was aliquoted in 100 pmol (20 pmol/μl). The therapeutic gene was amplified by PCR and the product was purified by silica based gel extraction. The purified PCR product (3–5 μg) was ligated into peptide vector (20 pmol). The PCR product ligated with the peptide vector was purified by ethanol precipitation and it was rehydrated in phosphate buffered saline and then injected intravenously in two dogs with SLE-like disease.

2.4. Detection of therapeutic gene transcription

To detect transcription of the therapeutic gene in the experimental dogs, a pair of primers amplifying the fusion portion that does not exist in normal dogs was prepared: 5′-ACAAAGTGAACCTCACCATC-3′ and 5′-ACACTGGACACACTGTAGCA-3′. The PBMCs were isolated from approximately 3 ml of venous blood supplemented with 0.42 ml of CPDA as an anti-coagulant by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation. The isolated PBMCs were washed twice with PBS and subjected to total RNA isolation. The total RNA was isolated (Nucleospin® RNAII, Macherey-Nagel GmbH& Co. KG, Germany) and RT-PCR (Reddy Mix Reverse-iT One Step Kit, AB gene, UK) was performed following the manufacturer's instructions. For confirming that the vector delivered the gene to various tissues, therapeutic gene was injected

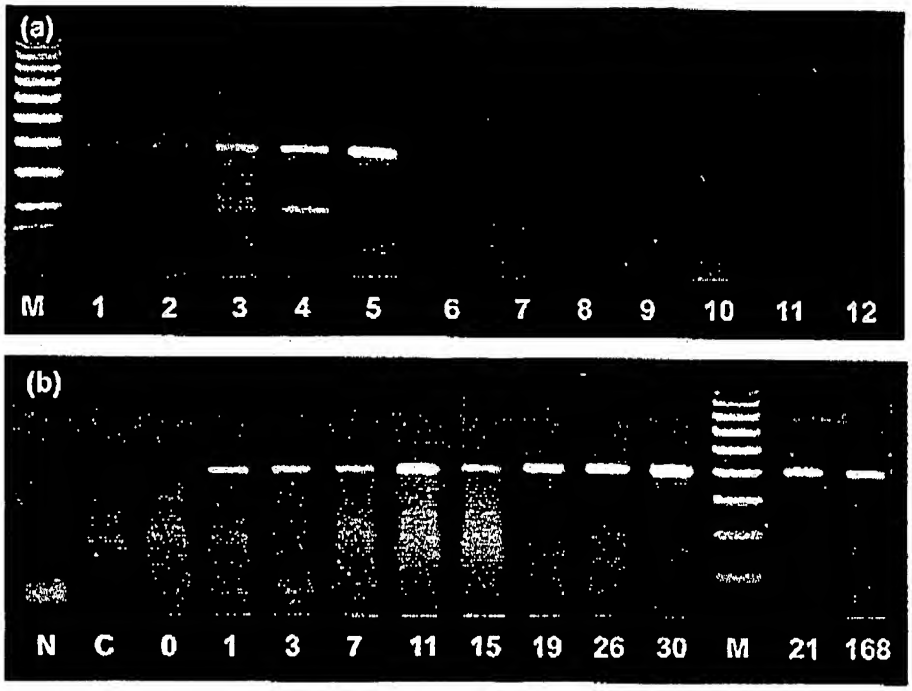

Fig. 2. RT-PCR to confirm the transcription of the therapeutic gene in rat tissues and dog PBMCs. (a) Expected size of 394 bp band size is shown. (M: 100 bp ladder, 1, 6: liver, 2, 7: kidney, 3, 8: spleen, 4, 9: lung, 5, 10: muscle, 11, 12: distilled water negative control, 1–5: gene injected rat, 6–10: PBS injected control rat.). (b) RT-PCR was carried out from PBMCs. A single band of 394 bp is shown (N: negative control, distilled water, C: negative control from non-treated dog; 0, 1, 3, 7, 11, 15, 19, 26, and 30 days after gene therapy, respectively, from treated dog 2, M: 100 bp ladder, 21 and 168 days after gene therapy, respectively, from treated dog 1).

intravenously or intraperitoneally into Sprague–Dawley rats (5 weeks old, female). Control rats were injected an equal volume of PBS intravenously. Total RNAs were prepared from the sacrificed rat tissues, which were obtained 3 days after injection by using of Trizol reagent. RT-PCR were performed with oligo-dT and the same specific primer used in dogs.

2.5. ELISA for detection anti-peptide vector antibodies

IgG antibodies to peptide vector was measured in the sera of control and treated dogs collected on 0, 3, 7, 15, and 30 days after gene therapy. Peptide vector (12.5 μg/well) was coated overnight at 4 °C in PBS. The excess binding sites were blocked with 1% BSA in PBS. Dog sera were tested at 1:200 dilution, and the amount of specific antibody bound to the peptide vector was

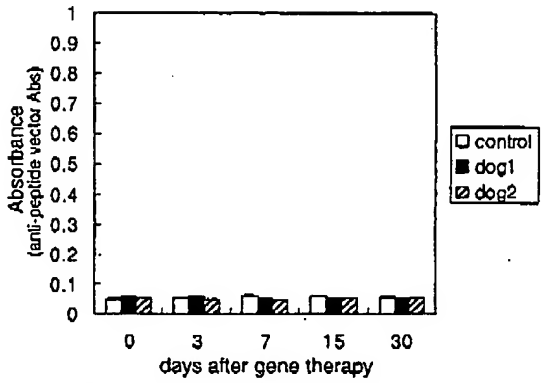

Fig. 3. Assay of anti-peptide vector antibodies. Antibody against the peptide vector was determined in dog sera on 0, 3, 7, 15, and 30 days after gene therapy. Compared to control, absorbances of anti-peptide vector antibodies in treated dogs were not significantly different.

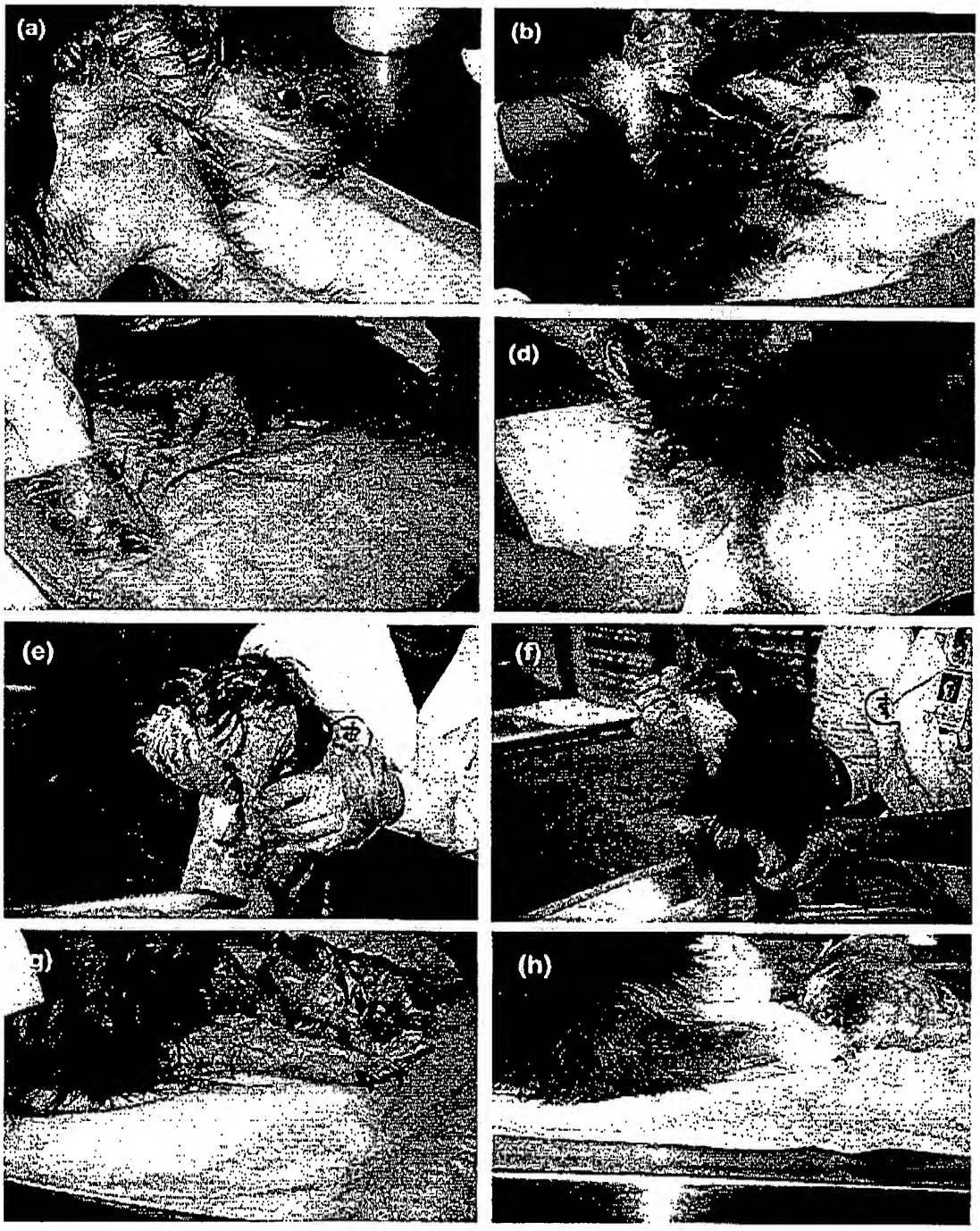

Fig. 4. Gross morphologic change in gene therapy. In experimentally-induced SLE dogs, severe alopecia was shown in the chest and head (a), the hindlimbs (c), the head and neck (e), the forelimbs (g). After gene therapy, the skin regained its normal gross morphologic features. Alopecia was not shown any more (b, d, f, and h).

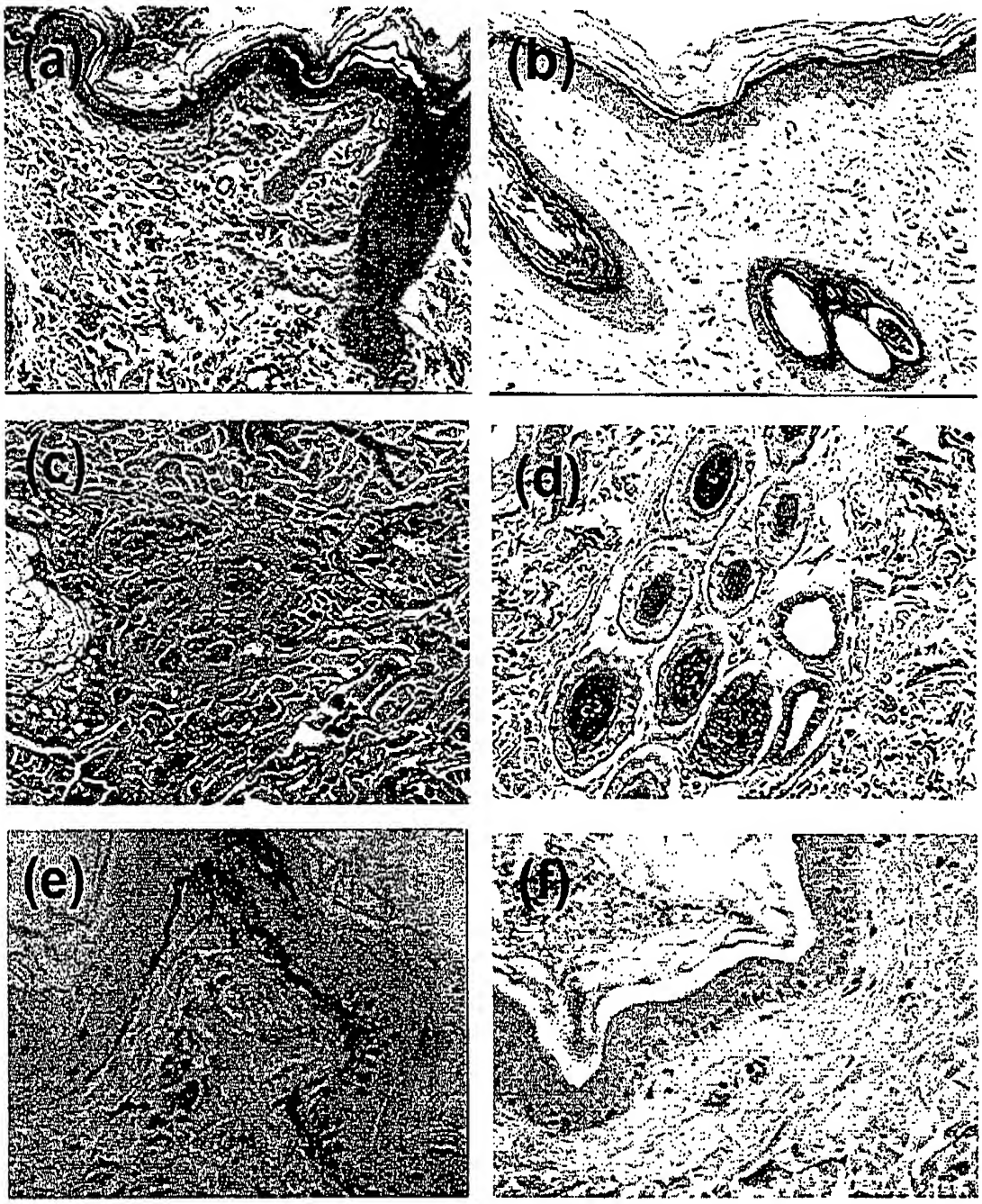

Fig. 5. Microscopic observation in gene therapy. Skin biopsy from the HS-immunized dog 12 weeks after the final immunization (a, c, and e) and after CTLA4Ig gene therapy (b, d, and f). (a) Orthokeratotic hyperkeratosis in the epidermis and perivascular lymphoplasmacytic infiltration in the superficial dermis (×400, H&E). (b) Note slight (negligible) superficial dermal infiltration of lymphocytes and plasma cells compared to that

determined using peroxidase-conjugated rabbit anti-dog IgG as secondary antibody and *o*-phenyllenediamine dihydrochloride (Sigma P9187) as substrate. Stop solution (3 M HCl) was added and color development was read at 492 nm using an automatic plate reader.

2.6. Urine protein and creatinine determination

Urine was collected by catheterization between 10 a.m. and 2 p.m. Urine protein was determined as described by Lott et al. (1983). Urine creatinine was determined by modified Jaffe reaction after diluting to 1:100 with deionized water. The urine protein to creatinine ratio (UP/C = urine protein (mg/dl)/urine creatinine (mg/dl)) is used to determine the extent of protein loss without the necessity of 24-h urine collection. A ratio of 0.6 or less indicates normal urine protein loss. A ratio of more than 1 indicates significant urine protein loss and a glomerular lesion (Sodikoff, 1995).

2.7. Histopathological examination

Skin tissues isolated from dogs at 12 weeks after the final HS immunization, and at 99 days after gene therapy were examined under a light microscope after H&E and immunostaining for immunoglobulins and C3. Heavy chain specific goat anti-dog IgG, μ chain specific goat anti-dog IgM, and goat anti-dog C3 (all from Bethyl laboratories, Montgomery, TX) were used as primary antibodies with each at 1:200 dilution. Horseradish peroxidase-anti-goat IgG(H + L) was used as secondary antibody at 1:200 dilution.

2.8. Fluorescence assay for autoantibodies in HS-immunized dogs

Indirect immunofluorescence for autoantibodies to nuclear and cytoplasmic antigens was performed using acetone-fixed CRFK cells as substrates. Dog sera were tested at 1:2–1:128 dilution, and binding of autoantibodies was detected using fluorescein isothiocyanate-conjugated goat anti-dog IgG (1:16 dilution). Fluorescence was observed with an epifluorescence microscope. Sera were obtained at 3, 7, 11, 18, 25, and 27 weeks after HS immunization, and at 7, 32, and 99 days after gene therapy.

3. Results

3.1. Construction of the therapeutic gene

The map of the therapeutic gene and vector are shown in Fig. 1.

3.2. Delivery of the therapeutic gene with the non-viral peptide vector

In rats, the delivered therapeutic gene with the peptide vector was transcribed in many tissues when injected intraperitoneally (Fig. 2a), demonstrating that the vector delivered the gene to various tissues. No antibody against the peptide vector was detected in dog sera on 0, 3, 7, 15, and 30 days after gene therapy in ELISA (Fig. 3). RT-PCR to detect transcription of the therapeutic gene was carried out from RNAs isolated from peripheral blood mononuclear cells (PBMCs) in the experimental dogs. As expected, a single band of 394 bp was seen in agarose gel electrophoresis (Fig. 2b), demonstrating that the transcription of the therapeutic gene lasted for at least 168 days after the injection.

3.3. Therapeutic effect

After CTLA4Ig gene therapy, cutaneous signs associated with SLE including alopecia, erythema, crusting, scaling and seborrhea resolved (Fig. 4a–h). In haematoxylin and eosin (H&E) staining, the superficial dermal infiltration of lymphocytes and plasma cells was remarkably reduced (Fig. 5a and b) and hair follicles showed telogen phase recovered to normal anagen phase (Fig. 5c and d). In immunohistological examination of skin from dogs treated with the therapeutic gene, the deposition of immunoglobulin M (IgM) (Fig. 5f) and C3 (data not shown) along the dermal–epidermal junction of the skins were negligible. The ANA titers were changed from >1:128

of non-treated skin (×400, H&E). (c) All of the hair follicles were severely atrophied showing telogen phase (×400, H&E). (d) The hair follicles recovered to normal anagen phase (×400, H&E). (e) Deposition of IgM along the dermal–epidermal junction is prominent (often known as a positive "lupus band", ×400, ABC). (f) Deposition of IgM in the dermal–epidermal junction is negligible (×400, ABC).

Table 1
Change of ANA titers in the SLE-induced dogs after gene therapy with non-viral peptide vector encoding canine CTLA4Ig gene

Treatment	Before treatment	Days after treatment		
		7	32	99
Treated dog	>1:128	1:64	1:2	1:2
Non-treated SLE dogs	>1:128	>1:128	>1:128	>1:128

Table 2
Change of urine protein-to-creatinine ratios (UP/Cs) in the SLE-induced dogs after gene therapy with non-viral peptide vector encoding canine CTLA4Ig gene

Dog	Before treatment	Days after treatment		
		7	32	99
Dog 1	2.62	1.03	0.64	0.58
Dog 2	1.51	0.99	0.27	0.35

to 1:2 (Table 1) and UP/Cs were reduced to normal ranges (Table 2).

4. Discussion

In this study, we intended to demonstrate two concepts; the possibility of the use of peptide vector and therapeutic effects in SLE. This study was the first study on gene therapy using a non-viral peptide vector.

SLE is a polyorgan immune-mediated disease, which commonly manifests non-erosive polyarthritis, glomerulonephropathy, hemolytic anemia, thrombocytopenia, neutropenia, polymyositis, persistent/recurring fever, and facial/mucocutaneous dermatitis (Halliwell and Gorman, 1989; Meyer et al., 1992; White, 2000). Among the numerous criteria to diagnose canine SLE that have been proposed, the most commonly accepted are that there should be hightitred serum ANA along with at least two of the autoimmune-associated clinical manifestations listed above (Day, 1999; Meyer et al., 1992; Paterson, 1998). In this study, all the HS-immunized dogs showed proteinuria, skin disease, and high-titred serum ANA. The HS-immunized dogs satisfied the criteria to diagnose canine SLE. The mechanism of glomerular injury in this model is assumed to involve deposition of preformed immune complexes (HS + anti-HS-Abs) from the circulation at various sites within the gromerular basement membrane (GBM). Alternatively, circulating antibody (anti-HS-Abs) directly bind to GBM. Therefore, it is possible to form an immune complex. Heparan sulfate could be one of the candidate autoantigens in SLE because it serves as a target antigen for in vivo cross-reactive anti-DNA antibodies (Suzuki et al., 1993). In our study, HS-immunization induced the increase of cross-reactive anti-nuclear Abs (ANA). Therefore, it is possible that during immunization with HS in CFA, the negatively charged HS could bind to nuclear materials released from dying cells, and thus elicit an anti-nuclear antibody response capable of binding both HS and nuclear materials. We expect that immune complexes such as 'nuclear materials + ANA' are produced and subsequently lodged in small vessels and the basement membrane zone of the skin and in various organ systems including the GBM of kidney.

Several kinds of viruses, including retrovirus, adenovirus, adeno-associate virus, and herpes simplex virus, have been manipulated for use in gene transfer applications. Although the process of transgene elimination may not be a problem in cancer or transplantation gene therapy, it hampers clinical applications such as the treatment of hereditary genetic disorders or neurodegenerative diseases, which require long-term transgene expression. Furthermore, the generation of adenovirus-neutralizing antibodies by the humoral arm of the acquired immune response, coupled with the likely pre-exposure to wild type adenovirus by most populations, means that re-administration of rAd is likely to be unsuccessful without prior tolerization (Dai et al., 1995). Recombinant adeno-associated vectors (rAAVs) also have the drawback of a limited packing capacity for the transgene (4.7 kb). As rAAVs are deleted of all viral genes, these vectors are believed to be relatively non-immunogenic. After in vivo gene transfer of rAAVs, no evidence of a cellular immune response has been shown; however, specific circulating antibodies to rAAVs have been detected (Xiao et al., 1996), limiting their potential re-administration. In herpes simplex virus vectors (HSVs), the problem of vector toxicity is currently an important limitation to their use (Laquerre et al., 1999; Lowenstein et al., 1994). Viral vectors internalize into cytoplasm, after binding to their specific extracellular receptor (Stone

et al., 2000). In contrast, our peptide vector may penetrate directly the cellular membrane. The N-terminal of the peptide vector is composed of aliphatic amino acids, and this portion easily infiltrates into the phospholipids bilayer of cellular membrane. The C-terminal amino acids of the vector have basic side chains, and these basic amino acids interact with negative charges of the inner cellular membrane. We believe that the ionic strength increases the power of penetration across the cellular membrane.

In this study, the therapeutic gene was expressed for at least 168 days in vivo from the injection. We think that the long duration of the expression is attributable to the peptide of vector blocking the activity of exonuclease. In the rat, delivered gene with peptide vector was expressed in various tissues. The result of rats treated intravenously was the same as that of rats treated intraperitoneally. Therefore, tissue distribution was not different between the routes of administration. We didn't determine the tissue distribution of the vector in dogs. Because we treated the therapeutic gene to only two dogs, we didn't kill them to investigate how long the delivered therapeutic gene with the peptide vector is transcribed. Instead, we used rats to determine if the therapeutic gene was transcribed in various tissues. If gene therapy on SLE dogs was not successful, it is worth to see if tissue distribution is different among species. Because gene therapy on canine SLE model was successful and the therapeutic gene was transcribed in canine PBMCs (as confirmed by RT-PCR amplification), we believe that the tissue distributions of the therapeutic gene in dogs and rats are the same. These characteristics, delivering the therapeutic gene to various tissues and long expression, may overcome the limitation of viral vectors in gene therapy. Antibody to canine CTLA-4 protein was not detected and that of human and murine have no-cross reactivity with canine CTLA-4. So we did not determine the level of CTLA-4 protein but we showed that the therapeutic gene was expressed in treated animal by RT-PCR.

One of the main drawbacks of viral vectors is the immune response elicited against the viral vector and the vector-encoded protein that infected cells express (Cartmell et al., 1999). This immune response can be separated into two phases, comprising an early innate inflammatory response and a later acquired immune response (Stone et al., 2000). In this study, acute immune responses as clinical signs associated with inflammation were not detected and CBC were normal. Because the size of the leader peptide is as small as a hapten, it is not expected to evoke immune responses in the hosts. And the rest of the construct is DNA, so normal host's immune system will recognize it as a self. Actually, no antibody against the peptide vector was detected in dog sera on 0, 3, 7, 15, and 30 days after gene therapy in ELISA. Since CTLA4Ig can inhibit antibody formation, further study is needed to confirm if the peptide vector encoding other genes invokes host immune response. We didn't determine whether the treated dogs make an immune response to the fusion protein. But fusion protein is composed of canine CTLA-4 and the CH2–CH3 domains of canine IGHAC. Therefore, we expect that dogs' immune system recognize the fusion protein as self, and thus, no immune response to the fusion protein is occurred in the dogs. In addition, because the CTLA4Ig can inhibit antibody formation, we expect that dogs won't present an immune response to the fusion protein. We didn't evaluate the effect of long-term expression of the transgene on normal immune function in dogs treated with the therapeutic gene. However, in this study, it was demonstrated that the treated dogs has normal body conditions based on CBC, serum biochemistry and urinalysis data. As the peptide vector does not use the packaging mechanism of a virus, it is not expected to have size limitation. Since the peptide vector does not include integrase, it will not integrate into the host's genome. In the present study, we showed that the peptide vector has most of the features of an ideal vector.

In this study, we showed that the gene therapy using the non-viral peptide vector which encoding canine CTLA4Ig gene was very effective in the canine SLE model. The strategy to control T cell activity using CTLA4Ig gene construct can be applied to treat other kinds of autoimmune diseases and asthma, and to inhibit graft rejection and graft versus host disease. The study will give hope to patients who are suffering various genetic diseases and the diseases resulting from hypersensitivity. Further study is needed to determine the level of CTLA-4 protein expression, to increase the number of experimental animal and to elucidate the characteristics of the peptide vector.

Here, we propose the application of our peptide vector to treat SLE in dogs and our results encourage

the general application of the non-viral peptide vector to various gene therapies.

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